THE INFLUENCE OF THE SINUS GLANDS ON GASTROLITH FORMATION IN THE CRAYFISH

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Introduction

The sinus glands located within the eyestalks of crustaceans have long been known to have an endocrine function in regulating pigmentary effectors. These glands, first described by Hanström (1933), have been the subject of various studies. But even before the sinus glands were known, there was evidence that the eyestalks formed a chromatophore-activating substance.

Decrease of the intermoult period after eyestalk removal seems to be hormonal in nature although the evidence in support of this is not yet complete. Megušar (1912) found that ecdysis occurred at more frequent intervals in eyestalkless than in normal *Astacus*. Brown and Cunningham (1939) made similar observations on the crayfish, *Cambarus immunis*, and Abramowitz and Abramowitz (1940) and Kleinholz and Bourquin (1941) on *Uca pugilator*. Smith (1940) showed that removal of both eyestalks in young crayfish shortened the intermoult period by slightly more than 30 per cent. Darby (1938) states that operative injury hastens the onset of moult in *Crangon armillatus*, but Smith (1940) showed that this was not true in young *Cambarus clarkii*. Plankemann (1935) reported that such diverse factors as starvation, increased metabolism, color of background and pH of seawater might possibly affect the rate of moulting.

Since moulting and calcium metabolism are closely related phenomena, it might be expected that the mobilization and transport of calcium would also be regulated by a similar hormonal factor. Koller (1930) found that calcium in the moulted exoskeleton of *Crangon*, after eyestalk removal, was less than in the casts of normal animals and concluded that the so-called eyestalk hormone was definitely associated with calcium metabolism. Plankemann (1935) drew a similar conclusion from the known hormonal control of moulting in insects and the fact that the calcium content of the crustacean skeleton was modified during ecdysis.

Brown and Cunningham (1939) are the only ones so far to have tested the possibility of hormonal control of ecdysis in crustaceans by implantation of sinus glands after eyestalk removal. They found that the removal of the eyestalks increased the rate of moulting. When sinus glands were implanted into eyestalkless animals, there was a slight lengthening of the intermoult period. Low survival of their animals complicated their experiments so that while an endocrine factor in the regulation of ecdysis was indicated, conclusive evidence for the view was lacking.

In several of the Astacura gastroliths occur. They are rounded prominences, one on each side of the anterior region of the cardiac stomach, which are found in *Cambarus virilis* fully developed in the late spring.[1] The outer or lateral surface of each gastrolith is in contact with the epithelium of the stomach wall while the cuticular stomach lining covers the inner or medial surface. The outer surface of the gastrolith is smooth and convex, while the inner surface is flat.

Upon examination, a gastrolith can be seen to be composed of thin superimposed layers, the inner layers being parallel to the flat inner surface while the outer ones are concentric with the convex outer surface. A gastrolith is similar in structure to other parts of the exoskeleton except that the thicker layers are next to the epithelium.

During the moult the "stones" are freed by the casting off of the cuticular lining and are ground up and dissolved in the stomach, a new cuticle having been formed before shedding. According to Huxley (1889), in *Astacus fluviatilis* which are four years old, gastroliths begin to form forty days before ecdysis. The period is shorter in younger crayfish, being not more than ten days the first year after hatching. The process of their absorption takes twenty to thirty hours in young animals and seventy to eighty in the adult. Unless they are developed and then absorbed, ecdysis is not normally effected and the animal dies in the course of the process. Maluf (1940) states that gastroliths have about 3.16 per cent of the total ash present in the hard shell and are therefore of no significance after moulting. Numanoi (1939) describes the blood as more viscous and milky both during gastrolith formation and dissolution.

In an attempt to study the effect of the sinus glands on moulting in the crayfish, the gastroliths were most useful in determining oncoming moults, because their appearance precedes ecdysis and also since they are so easily detected by gross examination.

[1] Normal *Cambarus virilis*, collected in April and kept in the laboratory in running water, were observed to show a great burst of moulting in late May and early June. Of 75 of these animals, 38 moulted between May 27 and June 7.

The present investigation has been carried out with the hope of ascertaining more exactly the effect the sinus glands may have upon the process of ecdysis and especially of gastrolith formation in the crayfish. By the classical method of first removing the suspected gland and then reimplanting it, an attempt has been made to find what effect the gland has upon the formation of gastroliths which are indicative of oncoming moults.[2]

Material and Methods

In most instances observations were made on *Cambarus virilis;* however, *Cambarus clarkii* and *Cambarus immunis* were used in a few experiments. The specimens of *Cambarus virilis* were taken from local ponds and kept in running water in cement tanks except during experiments. The operated animals and controls were kept in some instances in individual finger bowls and in other instances in glass aquaria at room temperature (20–23° C.). The animals were not fed during experimental periods.

In removing the eyes, a thread was securely tied around the soft base of the eyestalk. With a small scalpel the part of the eyestalk just distal to the thread was severed. By this method bleeding was eliminated in most cases. At times some bleeding took place but a clot readily formed and the animals were not noticeably affected.

When a sinus gland was to be removed from an eyestalk in order to implant it into the same or another animal, the severed eyestalks were placed in Van Harreveld's (1936) crayfish perfusion fluid. The skeleton was split along the sides of the stalk by means of small bone forceps and the upper or dorsal part removed. This procedure exposed the sinus gland as a small bluish structure definitely differentiated from the mass of the eyestalk tissue. With the use of a dissecting microscope and fine needles it was relatively easy to disengage the gland and take it up in a pipette which was used in implanting the gland into the ventral abdominal region of the recipient animal.

In the histological study entire stomachs were removed and immersed in Bouin's fluid. They were rinsed in 70 per cent alcohol and then placed in 95 per cent and absolute alcohol. Cedarwood oil proved to be the best method of clearing before imbedding in paraffin. After sectioning they were stained with Mallory's triple stain and mounted.

Since the sinus glands, suspected of exerting hormonal control upon the moulting process in crayfish, are located in the eyestalks, the best

[2] This work was carried out under the direction of Dr. John H. Welsh, whose coöperation was most valuable.

approach seemed to be the removal of eyestalks from crayfish to determine whether their loss would cause the formation of gastroliths.

Eyestalk Ligation Experiments

In the first group of 29 *Cambarus virilis* both eyestalks were removed as mentioned above, the animals were killed after one or two weeks and their stomachs were examined. These experiments were started (using lots of 6 animals each) on September 28 and ran until December 7, 1940. In 25 of the 29 animals gastroliths were found, their size depending upon the weight of the animal and the length of time from operation to examination (Table I).

Table I

Animals with Two Eyestalks Removed

Eyes Off One Week	
Total number examined	12
Gastroliths present in	10
Average weight of animals	35.5 grams
Average weight of gastroliths (pair)	27.1 mg.
Eyes Off Two Weeks	
Total number examined	17
Gastroliths present in	15
Average weight of animals	19.9 grams
Average weight of gastroliths (pair)	10.1 mg.
Control Animals	
Total number examined	20
Number of animals found having gastroliths	none

In the second group one eyestalk was removed from each of 23 *Cambarus virilis* in the manner described above. These animals were killed after one or two weeks and their stomachs examined. In no instance was there any indication of gastroliths or signs that they were forming, as was the case in all normal controls. These experiments were started September 28 and ran through November 8, 1940.

Sinus Gland Transplantation Experiments

Since the removal of both eyestalks caused the animals to form gastroliths, it was decided to transplant sinus glands into eyestalkless crayfish to determine whether the glands might again exert their influence upon the animal's moulting process. If such were the case, these animals minus their eyestalks, and with implanted sinus glands should not form gastroliths.

In these experiments, all conducted between October 27, 1940 and March 7, 1941, the eyestalks were removed and two sinus glands implanted in the abdomen. In some instances the animals were examined one week after transplanting and in other instances additional transplants were made of two sinus glands at the beginning of the second week, and the animals were examined after two weeks. Twenty *Cambarus virilis* and 12 *Cambarus immunis* were used. Eleven of the animals died either on the day of the operation or the one following. Of those which survived, no traces of gastrolith formation were discernible upon examination of the stomachs.

Temperature Experiments

Through the fall and winter, in *Cambarus virilis*, eyestalk removal resulted in the formation of gastroliths. The question arose as to whether or not a low temperature would prevent gastrolith formation. It had been noted earlier by Smith (1940) that temperature had a marked influence on moulting in young eyestalkless crayfish (*Cambarus clarkii*).

Animals with their eyestalks removed were placed in aquaria in cold rooms set at various temperatures. Others were placed at room temperature (about 21° C.). They were examined after two or three weeks to determine the influence of temperature upon the formation of gastroliths. Tables II and III indicate the results.

In the experiments conducted in December and January minute gastroliths were found in only 5 out of 23 animals kept at 3° to 15° C., while all animals kept at room temperature had gastroliths at the end of two weeks. In the April–May experiments (Table III), due to the approaching spring moult, many of the animals had gastroliths present before eyestalk removal. (Assumed after examination of a number of normal crayfish most of which possessed small gastroliths.) Little, if any, increase in size of the gastroliths occurred, however, in the eyestalkless animals kept for three weeks at 3°, 5° and 15° C., while the crayfish which survived for three weeks at room temperature showed fully formed gastroliths. Thus it is seen that temperatures of 15° C. or lower prevent or at least slow up the formation of gastroliths induced by eyestalk removal. This suggests the possibility that an enzyme system is involved in the removal of calcium from the exoskeleton and its deposition as gastroliths and that this enzyme process is inhibited during the winter, in part by the low temperature, and in part by an inhibiting hormone from the sinus glands.

TABLE II

Showing animals with two eyestalks removed subjected to various temperatures (December–January)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
°C.		*in weeks*	*in grams*	*in mg.*
3	1	3	29.1	0
"	2	"	23.8	0
"	3	"	21.3	0
"	4	"	20.7	0
"	5	"	27.2	0
8	6	"	28.1	0
"	7	"	24.2	2.2
"	8	"	27.0	0
"	9	"	27.2	0
"	10	"	38.0	2.8
"	11	"	26.0	0.6
11	12	2	14.1	0.2
"	13	"	17.5	0
"	14	"	17.1	0.2
"	15	"	13.8	0
"	16	"	14.0	0
"	17	"	23.3	0
15	18	"	24.9	0
"	19	"	21.3	0
"	20	"	20.7	0
"	21	"	23.1	0
"	22	"	17.6	0
"	23	"	16.8	0
20–23	24	"	24.1	9.6
"	25	"	29.3	0.9
"	26	"	27.2	20.6
"	27	"	19.5	3.0
"	28	"	21.3	21.4
"	29	"	25.9	20.0
"	30	"	18.2	52.9
"	31	"	26.4	57.0
"	32	"	21.5	7.4
"	33	"	28.3	20.2
"	34	"	34.3	0.4
"	35	"	23.5	7.8
"	36	"	29.2	9.3
"	37	"	34.5	9.2
"	38	"	3.9	37.6
"	39	"	22.2	21.3
"	40	"	4.0	21.2

Histological Study

When it was seen that gastroliths were formed prior to moulting, whether this moulting were normal or induced by eyestalk removal, the question of the histological changes in the stomach epithelium prior to, and during gastrolith formation, arose. How soon after removal of

Table III

Showing animals with two eyestalks removed subjected to various temperatures (April–May)

Temperature	Animal	Experimental Period	Animal Weight	Gastroliths (pair) Weight
°C.		*in weeks*	*in grams*	*in mg.*
3	1	3	20.5	1.1
"	2	"	13.2	26.2
"	3	"	19.0	6.0
"	4	"	16.4	9.2
"	5	"	15.2	5.2
"	6	"	19.0	0
"	7	"	18.5	3.0
"	8	"	13.4	0.4
5	9	"	20.4	0
"	10	"	20.0	4.3
"	11	"	21.0	0.2
"	12	"	14.7	0
"	13	"	19.6	0
"	14	"	22.0	1.3
"	15	"	16.4	6.2
"	16	"	13.0	17.0
15	17	"	19.5	0.2
"	18	"	10.5	4.2
"	19	"	14.2	8.1
"	20	"	14.9	17.0
"	21	"	15.0	2.3
"	22	"	12.5	0
"	23	"	13.2	5.0
"	24	"	12.0	4.0
20–23	25	"	12.0	199.0
"	*26	"	11.0	141.0

* Four animals in this group died before the end of three weeks.

sinus glands is there a noticeable change in the stomach epithelium which secretes the gastroliths? What are these changes?

Regions of the stomach where gastroliths form were fixed and studied histologically. Figure 1 indicates the condition of the stomach lacking gastroliths. Figure 2 illustrates the epithelial changes observa-

ble in the same region when a gastrolith is starting to form. Twenty-four hours after eyestalk removal there is proliferation of the epithelial cells (Fig. 2) which were originally of a low columnar type (Fig. 1). Gradually these cells become tall columnar cells. There is a subsequent crowding of the sub-epithelial connective tissue. By the end of 48 hours the cells are tall, crowded together and show large prominent nuclei (Fig. 3). Sections made of stomachs of animals whose eyes had been removed for one or two weeks show much the same condition of the

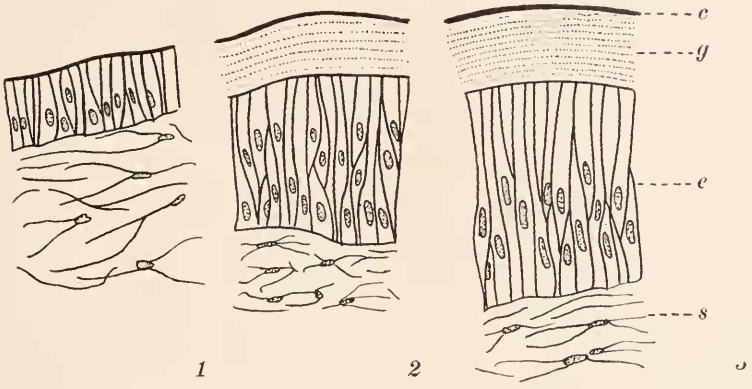

Fig. 1. Section of normal stomach of *Cambarus virilis* in region where gastrolith will form.

Fig. 2. Section showing gastrolith formation in stomach of *Cambarus virilis* 24 hours after eyestalk removal.

Fig. 3. Section showing gastrolith formation in stomach of *Cambarus virilis* 48 hours after eyestalk removal.

Abbreviations: *C*, cuticle; *G*, gastrolith; *E*, epithelium; *S*, subepithelial connective tissue.

epithelium as is illustrated by Fig. 3. Gastroliths begin to form 24 hours after eyestalk removal. However, Bouin's fluid containing acetic acid dissolves much of the inorganic material of the gastroliths and makes a true histological picture difficult to reconstruct.

Discussion

During the fall and winter normal animals show no gastroliths. Induced gastroliths, that is, those formed after eyestalk extirpation, were never found to be as large as those present in the spring and early summer during the normal moulting period. This was apparently due in part to the fact that gastroliths are from three to four weeks in their formation (forming much of their thickness during the last week) while

the experimental eyestalkless animals were ordinarily killed after a period of one or two weeks.

According to Robertson (1941) members of the suborders Anomura and Astacura do not appear to store calcium in the hepatopancreas. In Astacura gastroliths are formed in the foregut prior to moult. The gastroliths represent material which has been resorbed from the skeleton. A few hours after moulting they decrease in size and finally disappear, apparently being used as a source of calcium for the new skeleton.

Several investigators have studied the relation between calcium and moulting in Crustacea. The gastroliths, when present, and hepatopancreas, have been suspected as internal reserves drawn upon as the new skeleton is formed. Hecht (1914) found that once the skeleton of *Callinectes* was cast, the amount of calcium in the new individual was insufficient for the rebuilding of the new skeleton. Kleinholz, with the assistance of Emma Bourquin (1941), found that *Uca pugilator,* killed within five minutes after ecdysis, contained calcium equal to 1 per cent of the dry body-weight. The internal reserve constituted only 6 per cent of the total calcium content of the normal intermoult crab. The animal absorbed the balance of its calcium after moulting. Paul and Sharpe (1916) reported that in decapod Crustacea calcium was withdrawn from the hepatopancreas after ecdysis, and after the new shell was hardened, calcium was almost absent from this gland. Numanoi (1939) found the gastroliths of *Sesarma haematocheir* enlarged as moulting approached and disappeared after the moult. These changes he observed to take place concurrent with periodic changes in the blood calcium level.

Apparently the sinus gland acts as an agent controlling the passage of calcium, previous to moulting, from the exoskeleton into the blood where some of it is conveyed and deposited in the forming gastroliths. These act as reserve calcium which is utilized by the animal in order to harden partially its new skeleton until it can absorb more from its food and surroundings. Numanoi (1939) found that, in a species of *Sesarma,* when the calcium reserve is being dissolved after moulting, prior to being reprecipitated in the integument, blood calcium is raised to over forty times its normal level.

Why the sinus glands, which during the fall and winter inhibit moulting, do not do so in the spring is an interesting question. Apparently there is a seasonal variation in their activity which in the spring may be checked or inhibited by some other mechanism; also the low winter temperature would play some part in inhibiting gastrolith formation.

That the sinus glands are concerned in calcium metabolism of the crayfish during its moulting activity seems now to be reasonably certain.

The close relationship between the transfer of reserve calcium and moulting tends to point toward one mechanism as a control of both processes. The more intimate details of this mechanism still remain to be worked out.

Summary

1. Removal of both eyestalks of *Cambarus virilis* and *Cambarus immunis* induces the formation of gastroliths at times of the year when they would not normally form. Removal of one eyestalk fails to induce gastrolith formation.

2. Transplantation of sinus glands into the abdominal region of eyestalkless crayfish prevents the formation of gastroliths.

3. Temperatures of 15° C. and lower prevent or greatly retard the formation of gastroliths in eyestalkless crayfish.

4. As early as twenty-four hours after eyestalk removal marked histological changes occur in the regions of the stomach which secrete the gastroliths. There is proliferation and increase in height of the epithelial cells and they have already begun the secretion of the gastroliths.

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